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BRIEF DEFINITIVE REPORT

# G $\alpha_s$ -coupled receptor signaling and sleep regulate integrin activation of human antigen-specific T cells

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Efficient T cell responses require the firm adhesion of T cells to their targets, e.g., virus-infected cells, which depends on T cell receptor (TCR)-mediated activation of  $\beta_2$ -integrins. G $\alpha_s$ -coupled receptor agonists are known to have immunosuppressive effects, but their impact on TCR-mediated integrin activation is unknown. Using multimers of peptide major histocompatibility complex molecules (pMHC) and of ICAM-1—the ligand of  $\beta_2$ -integrins—we show that the G $\alpha_s$ -coupled receptor agonists isoproterenol, epinephrine, norepinephrine, prostaglandin (PG) E<sub>2</sub>, PGD<sub>2</sub>, and adenosine strongly inhibit integrin activation on human CMV- and EBV-specific CD8<sup>+</sup> T cells in a dose-dependent manner. In contrast, sleep, a natural condition of low levels of G $\alpha_s$ -coupled receptor agonists, up-regulates integrin activation compared with nocturnal wakefulness, a mechanism possibly underlying some of the immune-supportive effects of sleep. The findings are also relevant for several pathologies associated with increased levels of G $\alpha_s$ -coupled receptor agonists (e.g., tumor growth, malaria, hypoxia, stress, and sleep disturbances).

## Introduction

The initiation and execution of efficient T cell responses require the recruitment of T cells to lymphoid and nonlymphoid tissues (Ley et al., 2007), as well as the formation of immunological synapses with antigen-presenting cells (APCs) or target cells (such as virus-infected or cancer cells; Scholer et al., 2008; Dustin and Long, 2010; Fooksman et al., 2010). The modulation of T cell adhesiveness by regulation of integrin activation is crucial to these steps. Recirculating T lymphocytes express high levels of membrane-bound  $\beta_2$ -integrins (Dimitrov et al., 2009, 2010), which are maintained in a nonadhesive (inactive) state (Evans et al., 2009). Immediate activation (i.e., increase in affinity and avidity) of  $\beta_2$ -integrins induced by chemokines allows the arrest of T cells on the endothelium and their subsequent extravasation into tissues (Ley et al., 2007). A similar activation of  $\beta_2$ -integrins in response to TCR engagement by cognate peptides presented by MHC molecules (pMHC) on APCs or target cells is also required for the formation of stable immunological synapses (Dustin and Springer, 1989; Dustin and Long, 2010; Fooksman et al., 2010; Long, 2011).

Research on the regulation of integrin-mediated adhesion has focused over the past 35 yr exclusively on pro-adhesive signals, such as chemokines and pMHC. Only recently, the existence of anti-adhesive factors, such as G $\alpha_s$  (a heterotrimeric G protein subunit that activates the cAMP-dependent pathway)-coupled receptor agonists, nitric oxide, and carbon monoxide has become evident (Chigaev et al., 2008, 2011a,b, 2014). Specifically, it has been shown in monocytes that the chemokine-induced integrin affinity is down-regulated by anti-adhesive signaling derived from G $\alpha_s$ -coupled receptor agonists like amthamine (H<sub>2</sub>-histaminergic receptor agonist) and isoproterenol ( $\beta_{1/2}$ -adrenergic receptor [AR] agonist; Chigaev et al., 2008, 2011b). The recruitment of cytotoxic leukocytes to the blood during daytime and acute physical or psychological stress has been suggested to be mediated by epinephrine (a  $\beta_{1/2}$ -AR agonist), which induces a down-regulation of integrins, resulting in the de-adhesion of the cells from the endothelium of the marginal pool (Dimitrov et al., 2009, 2010). However, nothing is known about the effect of epinephrine or other G $\alpha_s$ -coupled receptor agonists on TCR-

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mediated integrin activation and formation of immunological synapses.

Several signaling molecules, including catecholamines (Wahle et al., 2005), prostaglandins (PGs; Scher and Pillinger, 2009; Kalinski, 2012), adenosine (Hoskin et al., 2008), dopamine (Yan et al., 2015), histamine, and serotonin (Kim et al., 2013) exert anti-inflammatory effects via their cognate  $G_{\alpha_s}$ -coupled receptors. Given the common intracellular mediator cAMP, here we asked whether these substances also share anti-adhesive properties. Sleep is known as a condition characterized by low levels of endogenous  $G_{\alpha_s}$ -coupled receptor agonists such as catecholamines (Dimitrov et al., 2015), PGs (Haack et al., 2009), and serotonin (Davies et al., 2014). We therefore additionally used sleep as an *in vivo* readout to assess effects of low levels of  $G_{\alpha_s}$ -coupled receptor agonists on adhesive properties of antigen-specific T cells in a physiological condition. In addition, because of the strong circadian rhythm in the levels of catecholamines (Dimitrov et al., 2015), PGs (Kamperis et al., 2004), serotonin (Davies et al., 2014), and adenosine (Chagoya de Sánchez et al., 1983), with a nadir during the rest phase, adhesion was measured across an entire day to detect a possible circadian rhythm of this parameter. For these purposes, we recruited healthy humans seropositive for CMV, because this chronic latent infection is characterized by a high number of antigen-specific T cells, allowing for the analysis of different T cell subsets. Adhesive properties of the cells were assessed by a new flow cytometry-based assay using soluble pMHC multimers for staining and activation of the antigen-specific T cells, and fluorescent intercellular adhesion molecule (ICAM)-1 multimers (mICAM-1) for visualization of activated  $\beta_2$ -integrins (Dimitrov et al., 2018).

We show that catecholamines,  $PGE_2$ ,  $PGD_2$ , and adenosine potently inhibit TCR-mediated integrin activation on human antigen-specific CD8<sup>+</sup> T cells, even at low (physiological) concentrations, whereas nocturnal sleep up-regulates integrin activation by reducing  $G_{\alpha_s}$ -coupled receptor signaling. Our findings support the view that conditions of low levels of  $G_{\alpha_s}$ -coupled receptor agonists, as generated during sleep, favor the formation of immunological synapses and thereby enhance T cell responses.

## Results and discussion

### Epinephrine potently inhibits pMHC multimer-induced activation of $\beta_2$ -integrins on antigen-specific CD8<sup>+</sup> T cells

We have recently developed a whole blood flow cytometry-based assay to monitor  $\beta_2$ -integrin activation on antigen-specific CD8<sup>+</sup> T cells by staining the cells with mICAM-1 upon antigen-specific stimulation (Dimitrov et al., 2018). Up-regulation of integrin activation on T cells occurs within a few minutes after TCR engagement and is, so far, the earliest measurable correlate of T cell functionality (as measured by cytokine production and expression of cytotoxic markers) following stimulation (Dimitrov et al., 2018). Thus, this assay is optimal for detecting acute effects of endogenous, fast-acting substances like catecholamines on T cell function.

We first tested the effect of epinephrine at very low ( $10^{-10}$  M), stress/exercise-like ( $10^{-9}$  M), and high ( $10^{-8}$  M; pathophysiological,

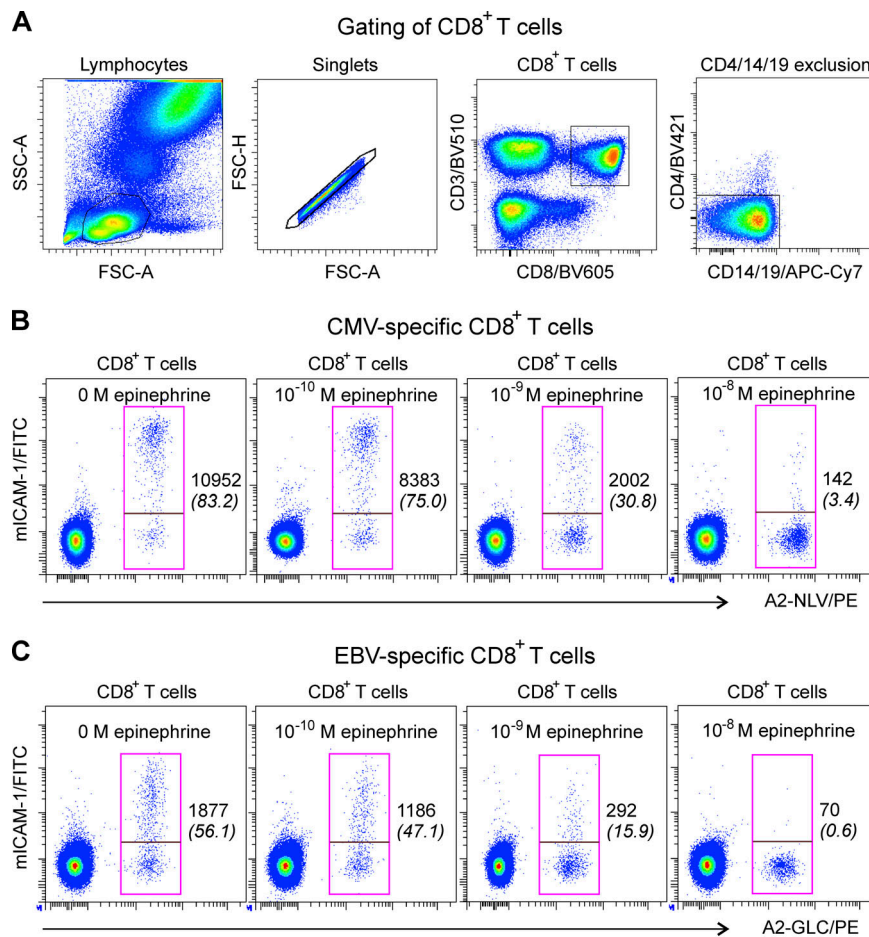
such as during hypoglycemia) concentrations on integrin activation. Whole blood from five HLA-A2<sup>+</sup> and CMV-seropositive donors was coincubated with A2/NLV (CMV) or A2/GLC (EBV) pMHC multimers and mICAM-1 for 5 min. The gating strategy to identify CD8<sup>+</sup> T cells is shown in Fig. 1 A. As previously shown (Dimitrov et al., 2018), more than two-thirds of A2/NLV<sup>+</sup> cells and approximately 30% (with a large inter-individual variability) of A2/GLC<sup>+</sup> cells were mICAM-1<sup>+</sup> (left plots on Fig. 1, B and C, respectively, shown for one representative CMV- and EBV-seropositive donor; and Table 1). Preincubation of the blood cells for 5 min with epinephrine reduced  $\beta_2$ -integrin activation by one-fourth to one-third at the lowest concentration and by 80% at stress/exercise-like levels, and almost completely inhibited it at high concentrations (Fig. 1, B and C; and Fig. 2, A and B). Previous assays assessing the effect of  $G_{\alpha_s}$ -coupled receptor agonists on integrin activation in monocytes relied on cell lines or processed blood, thus not allowing analysis of fast and fine-tuning effects relevant for *in vivo* situations (Chigaev et al., 2008, 2011b). In contrast, costaining of whole blood cells with pMHC and mICAM-1 proved to be a simple and sensitive method for detecting fast changes in  $\beta_2$ -integrin activation on antigen-specific T cells induced by even very low physiological levels of epinephrine.

### $G_{\alpha_s}$ -coupled receptor agonists down-regulate TCR-induced integrin activation on CMV- and EBV-specific T cells

Next, we determined the effect and relative potency of different  $G_{\alpha_s}$ -coupled receptor agonists on the regulation of  $\beta_2$ -integrin activation. Isoproterenol, epinephrine, norepinephrine,  $PGE_2$ ,  $PGD_2$ , and adenosine down-regulated  $\beta_2$ -integrin activation in a dose-dependent and agonist-specific manner, whereas dopamine, histamine, and serotonin had no effect (Fig. 2).

The effective concentration generating 50% of the effect ( $EC_{50}$ ) for CMV-specific cells was  $\sim 2\times$  higher for epinephrine and  $200\times$  higher for norepinephrine compared with isoproterenol (Fig. 2 A and Table 1). This suggests that the effect is mediated by  $\beta_2$ -ARs (rather than by  $\beta_1$ - or  $\beta_3$ -ARs), as the relative degree of binding affinity to the  $\beta_2$ -AR and the impact on cAMP production following receptor activation follows the same order (isoproterenol  $\approx 2\times$  epinephrine  $\gg$  norepinephrine; Hoffmann et al., 2004). Indeed, the inhibitory effect of epinephrine and norepinephrine was entirely abolished by preincubation with an antagonist of the  $G_{\alpha_s}$ -coupled  $\beta_2$ -AR (Fig. S1). Reduction of integrin activation first became significant at a concentration of  $5 \times 10^{-11}$  M for isoproterenol ( $P = 0.031$ ), at  $10^{-10}$  M for epinephrine ( $P = 0.047$ ), and at  $10^{-8}$  M for norepinephrine ( $P = 0.016$ ). Almost complete inhibition was achieved at isoproterenol and epinephrine concentrations of  $10^{-8}$  M and at a norepinephrine concentration of  $10^{-6}$  M.

$PGE_2$  and  $PGD_2$  inhibited  $\beta_2$ -integrin activation with similar  $EC_{50}$  on CMV-specific cells (Fig. 2 C and Table 1). Both PGs achieved a maximal effect at 10,000 pg/ml. However,  $PGE_2$  was more effective at lower concentrations: reduction of integrin activation first reached significance at a concentration of 100 pg/ml for  $PGE_2$  ( $P = 0.046$ ) and 333 pg/ml for  $PGD_2$  ( $P = 0.044$ ). The inhibitory effect of  $PGD_2$  was completely blocked after preincubation with an antagonist of the  $G_{\alpha_s}$ -coupled  $PGD_2$  receptor  $DP_1$  (Fig. S1). The effect of  $PGE_2$  was reversed by blocking the



**Figure 1. Epinephrine potently inhibits pMHC multimer-induced  $\beta_2$ -integrin activation on antigen-specific CD8<sup>+</sup> T cells.** Human whole blood cells were incubated for 5 min at 37°C in the presence of pMHC multimers (A2-NLV/PE or A2-GLC/PE) and mICAM-1. **(A)** Representative density plots showing the gating strategy used for defining pMHC<sup>+</sup> CD8<sup>+</sup> T cells. From left to right, the lymphocyte gate (on the forward scatter area [FSC-A]/side scatter area [SSC-A] plot), the FSC-A/FSC height (FSC-H) duplet exclusion, the gating of CD3<sup>+</sup>CD8<sup>+</sup> cells, and the exclusion of CD4<sup>+</sup>, CD14<sup>+</sup>, and CD19<sup>+</sup> events are shown. **(B and C)** CMV-specific (B) and EBV-specific (C) CD8<sup>+</sup> T cells were stained with A2-NLV/PE or A2-GLC/PE multimers, respectively. Multimer binding to the TCR leads to activation of  $\beta_2$ -integrins (left), which was detected by staining with mICAM-1. Preincubation of the cells for 5 min with  $10^{-10}$  M (18 pg/ml),  $10^{-9}$  M (180 pg/ml), or  $10^{-8}$  M (1,800 pg/ml) epinephrine partially or completely blocked the activation of the  $\beta_2$ -integrins. Numbers indicate the MFI of mICAM-1 binding on pMHC<sup>+</sup> CD8<sup>+</sup> T cells, and italic numbers in brackets indicate the frequency of mICAM-1<sup>+</sup> cells among pMHC<sup>+</sup> CD8<sup>+</sup> T cells.

high-affinity  $G_{\alpha_s}$ -coupled PGE<sub>2</sub> receptor EP<sub>4</sub>, but not by blocking the low-affinity receptor EP<sub>2</sub> (presumably because we did not use very high levels of PGE<sub>2</sub> [1,000 pg/ml]).

Adenosine inhibited integrin activation when peripheral blood mononuclear cells (PBMCs) were used (EC<sub>50</sub>:  $9.6 \times 10^{-8}$  M; Fig. 2 E), but was ineffective when using whole blood (probably due to the fast reuptake of adenosine by erythrocytes and

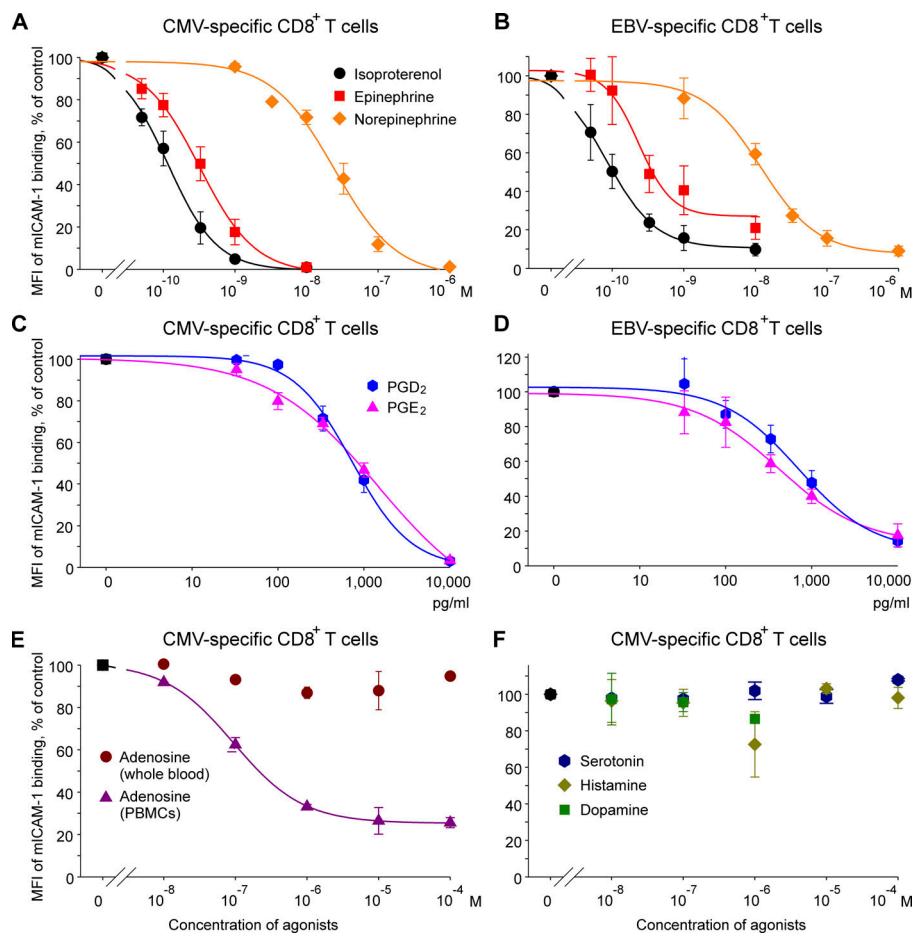
consequently extremely short half-life [ $<1$  s] of this substance in whole blood; Ramakers et al., 2008). The reduction of integrin activation became significant at the lowest dose used ( $10^{-8}$  M;  $P = 0.045$ ) and achieved a maximal effect at  $10^{-5}$  M. No effect was observed for the other  $G_{\alpha_s}$ -coupled receptor agonists tested (Fig. 2 F). Although there is some evidence for an immunomodulatory effect of serotonin, dopamine, and histamine, this

**Table 1. Characteristics of CMV- and EBV-specific cells and EC<sub>50</sub> values of  $G_{\alpha_s}$ -coupled receptor agonists**

	Total CMV-specific cells	Early differentiated CMV-specific cells	Intermediate differentiated CMV-specific cells	Late differentiated CMV-specific cells	Total EBV-specific cells
% of A2-NLV <sup>+</sup> or A2-GLC <sup>+</sup> $\pm$ SEM	100	32.8 $\pm$ 4.0	32.9 $\pm$ 5.1	30.6 $\pm$ 5.8	100
% mICAM-1 <sup>+</sup> $\pm$ SEM	83.0 $\pm$ 3.7	77.6 $\pm$ 4.6	83.5 $\pm$ 3.6	85.9 $\pm$ 5.0	30.3 $\pm$ 8.8
MFI of mICAM-1 <sup>+</sup> $\pm$ SEM	12,730 $\pm$ 1,904	8,459 $\pm$ 1,081	13,596 $\pm$ 1,796*	15,423 $\pm$ 3,198*	2,325 $\pm$ 988
EC <sub>50</sub> of isoproterenol, M	$1.4 \times 10^{-10}$	$1.27 \times 10^{-10}$	$1.47 \times 10^{-10}$	$1.58 \times 10^{-10}$	$9.9 \times 10^{-11}$
EC <sub>50</sub> of epinephrine, M	$3.9 \times 10^{-10}$	$3.20 \times 10^{-10}$	$4.15 \times 10^{-10}$ *	$3.94 \times 10^{-10}$ *	$2.5 \times 10^{-10}$
EC <sub>50</sub> of norepinephrine, M	$3.2 \times 10^{-8}$	$3.22 \times 10^{-8}$	$3.04 \times 10^{-8}$	$3.62 \times 10^{-8}$	$1.2 \times 10^{-8}$
EC <sub>50</sub> of PGE <sub>2</sub> , pg/ml	931	504	1,135*	1,073*	303
EC <sub>50</sub> of PGD <sub>2</sub> , pg/ml	797	606	737	810	952

\*,  $P \leq 0.05$  for comparisons between early versus intermediate or late differentiated CMV-specific T cells.





**Figure 2. Effect of  $G_{\alpha_s}$ -coupled receptor agonists on pMHC-induced  $\beta_2$ -integrin activation on antigen-specific CD8<sup>+</sup> T cells.** (A–F) Whole blood cells and (for adenosine) PBMCs were preincubated in the presence or absence of the indicated  $G_{\alpha_s}$ -coupled receptor agonists at the indicated concentrations for 5 min followed by staining with CMV A2-NLV/PE (A, C, E, and F) or EBV A2-GLC/PE (B and D) multimers and mICAM-1 for 5 min at 37°C. Means  $\pm$  SEM of the MFI of mICAM-1 binding are shown for the different agonists as percentage of the control (i.e., the sample without  $G_{\alpha_s}$ -coupled receptor agonists, which was set to 100%). The fitted standard curves were calculated by nonlinear regression;  $n = 5$  ( $n = 3$  for adenosine, serotonin, histamine, and dopamine).

finding can probably be explained by the lack or relatively low expression of intact  $G_{\alpha_s}$ -coupled receptors for these neurotransmitters on T cells (Saha et al., 2001a,b; León-Ponte et al., 2007; Kmiecik et al., 2012).

The inhibitory effect of  $G_{\alpha_s}$ -coupled receptor agonists does not seem to be limited to particular antigen-specific T cells, as we found similar results for EBV-specific CD8<sup>+</sup> T cells (Fig. 2, B and D; and Table 1). Here, reduction of integrin activation first became significant at a concentration of  $10^{-10}$  M for isoproterenol ( $P = 0.032$ ),  $3.3 \times 10^{-9}$  M for epinephrine ( $P = 0.032$ ),  $10^{-8}$  M for norepinephrine ( $P = 0.037$ ), and 333 pg/ml for PGE<sub>2</sub> and PGD<sub>2</sub> ( $P \leq 0.042$ ). Maximum inhibition was achieved at isoproterenol and epinephrine concentrations of  $10^{-8}$  M, at a norepinephrine concentration of  $10^{-6}$  M, and for both PGs at concentrations of 10,000 pg/ml.

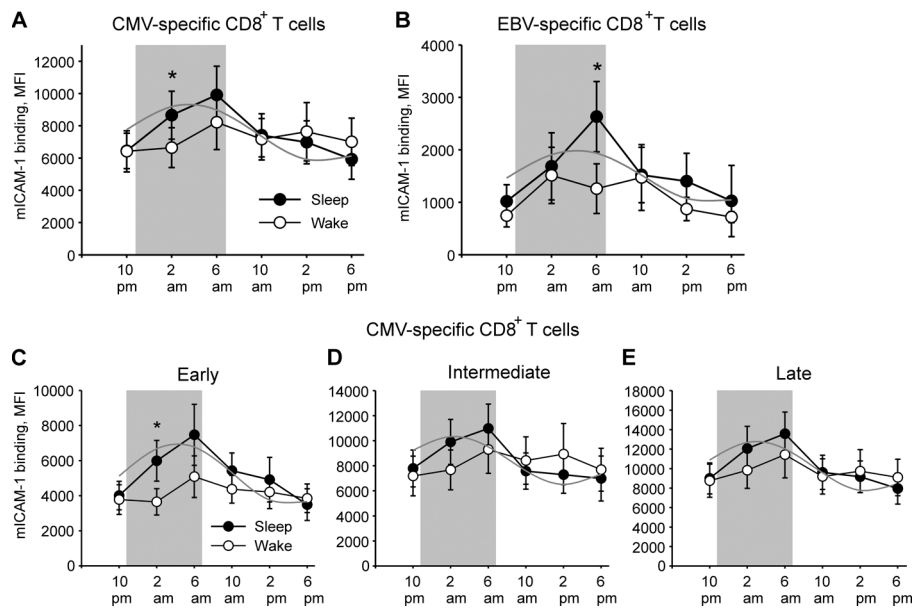
Catecholamines, PGs, and adenosine are known to directly suppress the production of cytokines and T cell proliferation (Wahle et al., 2005; Hoskin et al., 2008; Kalinski, 2012; Leone et al., 2015a). Our findings showing that these  $G_{\alpha_s}$ -coupled receptor ligands impair integrin activation reveal a new mechanism by which these molecules can exert immunosuppressive effects. Because the levels of  $G_{\alpha_s}$ -coupled receptor agonists needed to inhibit integrin activation *ex vivo* are observed in many physiological and pathological conditions, such as tumor growth, certain infections, hypoxia, and stress, these substances can be well expected to contribute to immunosuppressive effects

*in vivo* (Lokshin et al., 2006; Brudvik and Taskén, 2012; Kalinski, 2012; Leone et al., 2015a,b).

#### **$G_{\alpha_s}$ -coupled receptor agonists down-regulate TCR-induced integrin activation depending on the differentiation status of CMV-specific T cells**

We then tested whether mICAM-1 binding and the effects of  $G_{\alpha_s}$ -coupled receptor agonists depend on the differentiation status of antigen-specific T cells. As shown previously (Appay et al., 2002, 2008), CMV- and EBV-specific CD8<sup>+</sup> T cells can be separated into three differentiation subsets: early (CD27<sup>+</sup>CD28<sup>+</sup>), intermediate (CD27<sup>+</sup>CD28<sup>-</sup>) and late (CD27<sup>-</sup>CD28<sup>-</sup>) differentiated cells, based on their CD27 and CD28 expression. For CMV-specific cells, percentages of the three subpopulations were similar (Table 1 and Fig. S2 A), whereas EBV-specific cells were highly enriched in the early differentiated subset ( $70.3\% \pm 7.1\%$ ).

The mean fluorescence intensity (MFI) of mICAM-1 binding was higher on intermediate and late differentiated compared with early differentiated cells (Table 1 and Fig. S2 B). The inhibition of integrin activation by epinephrine and PGE<sub>2</sub> was strongest for the early differentiated cells, but there were no significant differences between subsets for the EC<sub>50</sub> of isoproterenol, norepinephrine, and PGD<sub>2</sub> (Table 1 and Fig. S2, C and D). The finding that epinephrine was more effective in reducing integrin activation in early compared with intermediate and late differentiated cells might be surprising, given that this subset



**Figure 3. Sleep increases  $\beta_2$ -integrin activation on antigen-specific CD8<sup>+</sup> T cells.** (A–E) Means  $\pm$  SEM of the MFI of mICAM-1 binding on total CMV-specific (A) and EBV-specific (B) CD8<sup>+</sup> T cells, as well as on early (CD27<sup>+</sup>CD28<sup>+</sup>; C), intermediate (CD27<sup>+</sup>CD28<sup>+</sup>; D) and late (CD27<sup>+</sup>CD28<sup>+</sup>; E) differentiated CMV-specific CD8<sup>+</sup> T cell subpopulations after staining with pMHC multimers and mICAM-1 are shown for a regular sleep–wake cycle (sleep, filled circles) and continuous wakefulness (wake, open circles). Gray lines illustrate adapted cosine curves for confirmed circadian rhythms in the sleep condition; the shaded area depicts bedtime. Significance is indicated for pairwise comparisons between conditions using paired *t* tests following significant ANOVA results. *n* = 10 for CMV; *n* = 5 for EBV; \*, *P* < 0.05.

expresses the lowest number of  $\beta_2$ -adrenoreceptors (Dimitrov et al., 2009, 2010). However, it is not uncommon that receptor expression levels do not correlate with the cell's responsiveness to a substance (Dimitrov et al., 2009), pointing to the presence of other factors, such as the molecules involved in the intracellular signaling cascade, that contribute to the magnitude of the substance effect as well.

#### Sleep increases $\beta_2$ -integrin activation on CMV- and EBV-specific CD8<sup>+</sup> T cells

Given that nocturnal sleep is characterized by low levels of  $G_{\alpha_s}$ -coupled agonists, such as catecholamines and PGs, we compared effects of sleep versus nocturnal wakefulness on  $\beta_2$ -integrin activation of CMV- and EBV-specific T cells. In a within-subject design, healthy CMV-positive participants spent one night in the sleep laboratory with sleep allowed between 11 pm and 7 am (sleep condition) and another night in which participants had to stay awake in bed during the same period (wake condition). Whole blood for the determination of integrin activation was collected repeatedly across an entire day, additionally enabling the detection of circadian rhythms.

Compared with the wake condition, sleep significantly increased the MFI of mICAM-1 binding on pMHC<sup>+</sup> CD8<sup>+</sup> T cells ( $F_{(1,9)} = 4.1$ ; *P* = 0.05 and  $F_{(1,8)} = 6.0$ ; *P* = 0.05 for sleep/wake  $\times$  period effect for CMV- and EBV-specific CD8<sup>+</sup> T cells, respectively; see Fig. 3, A and B, for comparisons at single time points). For CMV-specific T cells, the effect was evident already at 2 am (i.e., 3 h after the beginning of the sleep opportunity). Cosinor analysis confirmed a significant circadian rhythm in mICAM-1 binding on CMV- and EBV-specific cells during the regular sleep–wake cycle (*P*  $\leq$  0.023; Fig. 3, A and B), which was abolished in the wake condition (*P* = 0.07 and 0.10 for CMV- and EBV-specific cells, respectively). The fitted cosine curve for the sleep–wake cycle showed an acrophase (peak time) at 3:33 am  $\pm$  32 min and 4:15 am  $\pm$  50 min for CMV- and EBV-specific cells, respectively.

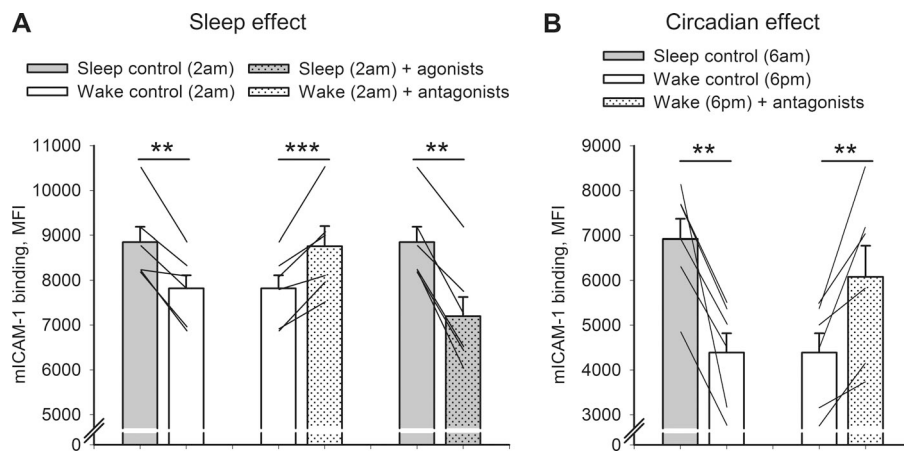
Our results demonstrate that a couple of hours of sleep loss suffice to reduce the adhesion capacity of antigen-specific

T cells. This finding shows that sleep has the potential to enhance the efficiency of effector T cell responses, which is especially relevant in light of the high prevalence of sleep disorders and conditions characterized by impaired sleep, such as depression, chronic stress, aging, and shift work (Akerstedt, 2003; Roth, 2007; Medina et al., 2014; Feinsilver and Hernandez, 2017).

#### Sleep selectively increases $\beta_2$ -integrin activation on early and intermediate differentiated CMV-specific CD8<sup>+</sup> T cell subsets

mICAM-1 binding at baseline (10 pm) across the sleep and wake conditions was lowest in early differentiated cells ( $3,889 \pm 570$ ), higher in the intermediate differentiated subset ( $7,479 \pm 1,055$ ), and highest in late differentiated cells ( $8,868 \pm 1,135$ ; *P*  $\leq$  0.003 for early vs. intermediate, early vs. late, and intermediate vs. late differentiated cells; Fig. 3, C–E). Compared with the wake condition, sleep significantly increased mICAM-1 binding on pMHC<sup>+</sup> CD8<sup>+</sup> T cells for the early and intermediate differentiated cells ( $F_{(1,9)} = 7.7$ ; *P* = 0.02 and  $F_{(1,8)} = 4.6$ ; *P* = 0.005 for sleep/wake  $\times$  period effect, respectively; see Fig. 3 for comparisons at single time points). Cosinor analysis confirmed a significant rhythm in mICAM-1 binding for all three subsets during the regular sleep–wake cycle (*P*  $\leq$  0.033), which descriptively was most pronounced for the early differentiated cells: with reference to peak values, mICAM-1 binding during nadir times was reduced by 33%, 30%, and 26%, respectively, in the three subpopulations. The fitted cosine curve showed an acrophase at 4:13 am  $\pm$  84 min for the early differentiated subset, at 2:19 am  $\pm$  76 min for the intermediate differentiated subset, and at 3:01 am  $\pm$  68 min for the late differentiated subset. Peak values of mICAM-1 binding (as derived from cosinor analyses) were significantly higher in the sleep condition compared with the wake condition (*P*  $\leq$  0.01), with a similar percentage change in all three subpopulations (57%, 56%, and 56%, respectively).

We could not measure integrin activation on undifferentiated (i.e., naive) T cells due to the inherent properties of the applied assay. However, because the early differentiated phenotype was the most responsive one, it is likely that adhesive properties of



**Figure 4. Regulation of integrin activation by plasma collected during sleep versus wakefulness is mediated by  $G_{\alpha_s}$ -coupled receptor signaling.** (A and B) Means  $\pm$  SEM of the MFI of mICAM-1 binding are shown on CMV-specific CD8<sup>+</sup> T cells incubated with plasma collected at 2 am during sleep versus wakefulness (A) and at 6 am (during nocturnal sleep) versus 6 pm (during daytime wakefulness; B). CMV-specific T cells were incubated with plasma collected at the indicated time points and conditions (sleep vs. wake) in the presence or absence of an antagonist composite (including  $\beta_2$ -AR, EP<sub>4</sub>, and DP<sub>1</sub> antagonists) or in the presence or absence of an agonist composite (including epinephrine, norepinephrine, PGE<sub>2</sub>, and PGD<sub>2</sub>). All experiments were performed in duplicates. Significance is indicated for pairwise comparisons between conditions or treatments using paired *t* tests. *n* = 6; \*\*, *P*  $\leq$  0.01; \*\*\*, *P* < 0.001.

naive T cells are also affected by  $G_{\alpha_s}$ -coupled receptors. Previous experiments have shown that sleep is essential for forming adaptive immune responses, which has been most impressively demonstrated by several studies using vaccination (Lange et al., 2003, 2011; Benedict et al., 2012). The mechanisms of this boosting effect of sleep on antigen-specific responses are not well understood. If our findings are indeed transferrable to naive T cells, an increased adhesion of naive T cells to APCs might at least partly explain the adjuvant-like effect of sleep on vaccination responses, thus revealing a novel mechanism underlying this effect.

#### Sleep regulates $\beta_2$ -integrin activation by suppression of $G_{\alpha_s}$ -coupled receptor signaling

Next, we tested whether the effect of sleep versus wakefulness on integrin activation is mediated by soluble factors via a  $G_{\alpha_s}$ -coupled receptor-dependent way. We collected heparin plasma from healthy participants on one night while they were asleep and on another night while they stayed awake in bed. We then incubated cells from a sleeping CMV-positive donor with the plasma collected during the two conditions (sleep vs. wake) in the presence or absence of  $G_{\alpha_s}$ -coupled receptor agonists or antagonists to compare integrin activation. These plasma experiments confirmed our findings on a sleep-induced increase in integrin activation: Incubation of CMV-specific cells with plasma collected at 2 am (i.e., the time point of significant differences between sleep and wakefulness in the foregoing sleep experiment) from sleeping participants significantly increased integrin activation on CMV<sup>+</sup> CD8<sup>+</sup> T cells compared with plasma collected at 2 am during nocturnal wakefulness (Fig. 4 A, left). We could also replicate our finding on a circadian rhythm observed in integrin activation by comparing effects of plasma collected at 6 am from sleeping participants versus plasma collected at 6 pm (during regular daytime wakefulness; Fig. 4 B, left).

Blocking catecholamines and PGs in the plasma derived from wake participants using a composite of  $\beta_2$ -AR, DP<sub>1</sub>, and EP<sub>4</sub> receptor antagonists (which were the antagonists shown to block

the effect of the respective receptor agonists; Fig. S1) enhanced integrin activation to levels comparable to the sleep condition (*P* < 0.01; Fig. 4). Adding a composite of catecholamines and PGs (epinephrine, norepinephrine, PGE<sub>2</sub>, and PGD<sub>2</sub>) at very low physiological concentrations mimicking sleep-wake differences (Haack et al., 2009; Dimitrov et al., 2015) to the plasma derived from sleeping participants significantly inhibited integrin activation (*P* < 0.01; Fig. 4 A, right). These results corroborate and add to our foregoing sleep experiment by proving that the effect of sleep deprivation is mediated by soluble factors present in the plasma and that these factors act via  $G_{\alpha_s}$ -coupled receptors. Even very low concentrations of  $G_{\alpha_s}$ -coupled receptors agonists that correspond to changes induced by a couple of hours without sleep significantly suppressed integrin activation, again demonstrating the great potency of endogenous  $G_{\alpha_s}$ -coupled receptor ligands to suppress T cell function.

#### General conclusions

We show here that several  $G_{\alpha_s}$ -coupled receptor ligands potently inhibit TCR-mediated integrin activation on antigen-specific CD8<sup>+</sup> T cells, whereas sleep up-regulates integrin activation by suppressing  $G_{\alpha_s}$ -coupled receptor signaling. Given the importance of integrin activation for the formation of immunological synapses (Dustin and Springer, 1989; Abram and Lowell, 2009; Dustin and Long, 2010; Fooksman et al., 2010; Long, 2011), our data suggest a critical role of conditions like sleep that are characterized by low levels of  $G_{\alpha_s}$ -coupled receptor ligands, in boosting T cell responses (Irwin et al., 1999; Lange et al., 2011). These findings provide a potential mechanism for some immune-enhancing effects of sleep on a new, so far unrecognized, level, namely the formation of immunological synapses by T cell adhesion to APCs or target cells. The findings are also important for understanding immunosuppression present in a variety of pathologies characterized by increased levels of adenosine (e.g., hypoxia, sleep apnea, and tumor growth), catecholamines (e.g., chronic stress and sleep disturbances), or PGs (e.g., tumor growth and malaria) (Kilunga Kubata et al., 1998; Hoskin et al., 2008; Sloan et al., 2010; Brudvik and



Taskén, 2012). Furthermore, the results suggest that  $G\alpha_s$ -coupled receptors of catecholamines, PGs, and adenosine might constitute immune checkpoint inhibitors, which could be manipulated for therapeutic purposes in various diseases, such as cancer (Leone et al., 2015b; Ohta, 2016; Ricon et al., 2019).

## Materials and methods

### Participants and study procedure

Ten physically and mentally healthy participants (five men and five women) were included in the study (mean age, 24.7 yr; range, 19–34 yr; mean body mass index, 22.4 kg/m<sup>2</sup>; range, 20–25 kg/m<sup>2</sup>). Participants were selectively recruited for being HLA-A2\* and CMV seropositive. Five of these individuals also had detectable levels of EBV-specific CD8<sup>+</sup> T cells, allowing us to quantify a second type of antigen-specific cells in this subsample. All participants were nonsmokers, did not suffer from sleep disturbances, and were not taking any medications (except for oral contraceptives in women) at the time of the experiments. None had a medical history of any relevant chronic disease or mental disorder. Acute illness was excluded by physical examination and routine laboratory investigation, including a chemistry panel, C-reactive protein concentration (<6 mg/liter), and a white blood cell count (<9,000/ $\mu$ l).

For investigation of the effect of sleep and the circadian system on adhesion properties of antigen-specific T cells, subjects participated in two experimental conditions, each starting at 8 pm and ending at 6 pm the following day, according to a within-subject crossover design. One condition (sleep) included a regular sleep-wake cycle, whereas in the other condition (wake), participants remained awake throughout the entire experimental period. In the sleep condition, sleep was allowed between 11 pm (lights off) and 7 am, whereas in the wake condition, participants stayed awake in bed in a half-supine position between 11 pm and 7 am. During this time they were listening to music and talking to the experimenter at normal room light. Both experimental sessions were separated by  $\geq 2$  wk for each participant. To control for a possible influence of the hormonal cycle in women, both experimental sessions took place within 3 to 6 d after the beginning of their menstrual cycle or the pill-free period.

On both conditions, blood was sampled at 10 pm, and then at 2 am, 6 am, 10 am, 2 pm, and 6 pm the next day. Blood was sampled via an intravenous forearm catheter, which was connected to a long thin tube and enabled blood collection from an adjacent room without disturbing the participant's sleep. To prevent clotting, ~500 ml of saline solution were infused throughout the night. The total volume of blood sampled during a session was 120 ml. Blood samples were always processed immediately after sampling. All participants spent one adaptation night in the sleep laboratory before the experimental sessions in order to become accustomed to the experimental setting.

For incubation of whole blood with various  $G\alpha_s$ -coupled receptor agonists, blood was collected at 6 am from five sleeping participants. We used blood from sleeping participants for these in vitro experiments, as endogenous  $G\alpha_s$ -coupled receptor agonists are lower during sleep than during wakefulness.

For the plasma experiments testing the  $G\alpha_s$ -coupled receptor dependence of the sleep/wake effect, we collected heparin plasma from six healthy participants at 2 am on one night while they were asleep and on another night while they stayed awake. Plasma was also sampled at 6 am during regular sleep and at 6 pm during regular daytime wakefulness for investigation of circadian rhythm effects. Indomethacin (a PG synthetase inhibitor; Sigma-Aldrich) was added to the plasma at a concentration of 10  $\mu$ g/ml before storage of the samples at  $-80^{\circ}\text{C}$  until further use. These six participants were not tested for CMV seropositivity, as only their plasma (but not the antigen-specific T cells) was used for further experiments (described in  $G\alpha_s$ -coupled receptor dependence of the effects of sleep...). Otherwise, the experimental procedures were comparable to the main sleep experiment. The study was approved by the Ethics Committee of the University of Tübingen, and all participants gave written informed consent.

### pMHC and ICAM-1 multimers

We produced biotinylated pHLA-A\*0201 monomers (CMV A2/NLVPMTATV [A2/NLV] and EBV A2/GLCTLVAML [A2/GLC]) in-house by conventional pMHC refolding, as previously described (Hadrup et al., 2015). We generated fluorescent pHLA-A\*0201 multimers by incubating monomers with streptavidin-PE or -APC (Thermo Fisher Scientific) at a 1:4 (streptavidin: pHLA-A2 monomer) molar ratio. Multimers were aliquoted and stored at  $-80^{\circ}\text{C}$  in Tris-buffered saline buffer containing 16% glycerol (Hadrup et al., 2015). The final concentration of azide was 0.035%.

We generated fluorescent ICAM-1-Fc/anti-Fc multimeric complexes by incubating 200  $\mu$ g/ml of recombinant human ICAM-1-Fc (produced as previously described; Bengtsson et al., 2013) with polyclonal anti-human Fc-FITC F(ab')<sub>2</sub> fragments (Jackson ImmunoResearch) at a 1:4 (ICAM-1-Fc:IgG part of anti-Fc-FITC fragments) molar ratio at  $4^{\circ}\text{C}$  for 3 h. We stored them in aliquots at  $-20^{\circ}\text{C}$  for  $\leq 1$  wk until use.

### Cell stimulation and mICAM-1 staining

We used a flow cytometry assay based on whole blood staining that we recently developed to monitor  $\beta_2$ -integrin activation on antigen-specific CD8<sup>+</sup> T cells (Dimitrov et al., 2018). This assay measures integrin activation by visualizing the specific cellular binding of mICAM-1, reflecting an increased affinity and/or avidity of  $\beta_2$ -integrins following TCR-induced activation. We incubated fresh heparinized whole blood (380  $\mu$ l/test) for 5 min at  $37^{\circ}\text{C}$  in a water bath in 5-ml Falcon tubes (BD Biosciences) with 0.6  $\mu$ g/ml CMV A2-NLV/PE or EBV A2-GLC/PE multimers and 6.25  $\mu$ g/ml mICAM-1/FITC. Immediately after incubation, we fixed the samples and lysed erythrocytes with FACS-Lysing solution (BD Biosciences) containing 1 mM Ca<sup>2+</sup> and 2 mM Mg<sup>2+</sup> for 5 min, followed by washing with PBS/0.5% albumin/0.1% sodium azide. After centrifugation, we stained the cells for surface markers in order to identify CD8<sup>+</sup> T cells and their subpopulations (early [CD27<sup>+</sup>CD28<sup>+</sup>], intermediate [CD27<sup>+</sup>CD28<sup>-</sup>], and late [CD27<sup>-</sup>CD28<sup>-</sup>] differentiated cells). The monoclonal antibodies CD4/BV421, CD3/BV510, CD8/BV605, CD28/PerCP-Cy5.5, CD27/APC, CD14/APC-Cy7, and CD19/APC-Cy7 (all from



BioLegend) were added to the cells for 15 min at room temperature. All antibodies were used at pretested optimal concentrations. The samples were acquired on a LSR Fortessa (BD Biosciences) and analyzed using FACS DiVa version 8.0. We collected at least 50,000 CD8<sup>+</sup> events for the antigen-specific assays. Results are presented as percentage of cells within the parent populations (only Fig. 1 and Fig. S2) or MFI of mICAM-1 binding on total pMHC<sup>+</sup> cells.

### In vitro effect of $G_{\alpha_s}$ -coupled receptor agonists and antagonists

To investigate the effects of  $G_{\alpha_s}$ -coupled receptor agonists on the TCR-induced  $\beta_2$ -integrin activation, whole blood taken at 6 am from five participants while asleep was preincubated for 5 min at 37°C in the presence or absence of (-)-isoproterenol ( $5 \times 10^{-11}$ – $10^{-8}$  M), (-)-epinephrine ( $5 \times 10^{-11}$ – $10^{-8}$  M), (-)-norepinephrine ( $10^{-9}$ – $10^{-6}$  M), PGD<sub>2</sub> (10–10,000 pg/ml), PGE<sub>2</sub> (10–10,000 pg/ml), dopamine ( $10^{-8}$ – $10^{-6}$  M; all from Sigma-Aldrich), adenosine ( $10^{-8}$ – $10^{-4}$  M), serotonin ( $10^{-8}$ – $10^{-4}$  M), or histamine ( $10^{-8}$ – $10^{-4}$  M; all from Carl Roth) before staining with pMHC multimers and mICAM-1 as described above. Due to the known rapid uptake of adenosine by erythrocytes when using whole blood (Ramakers et al., 2008), the assay for adenosine was also performed on PBMCs isolated in mononuclear cell preparation tubes (CPT; BD Biosciences) from three CMV-seropositive participants. In contrast to whole blood, PBMCs had to be stimulated with peptides (in addition to the pMHC multimers) to achieve optimal cell activation. Alterations in the MFI of mICAM-1 binding on the different in vitro drug treatments are presented as percentage of the control value (without  $G_{\alpha_s}$ -coupled receptor agonists), thereby eliminating interindividual differences in MFI.

To investigate whether the effects of the various ligands can be blocked by antagonists of the cognate  $G_{\alpha_s}$ -coupled receptors, whole blood was sampled at 9 am from three to four CMV-seropositive participants. The whole blood cells were then preincubated with the following antagonists at the indicated concentration before incubation of the cells with the agonists as described in Fig. S1:  $\beta_2$ -AR antagonist ICI-118,551 ( $10^{-7}$  M), PGE<sub>2</sub> EP<sub>4</sub> receptor antagonist ONO-AE3-208 ( $3 \times 10^{-6}$  M; both from Sigma Aldrich), PGE<sub>2</sub> EP<sub>2</sub> receptor antagonist PF-04418948 ( $3 \times 10^{-6}$  M), and PGD<sub>2</sub> DP<sub>1</sub> receptor antagonist MK-0524 ( $3 \times 10^{-6}$  M; Santa Cruz Biotechnology). Following 5 min of incubation, the cells were stained with CMV A2-NLV/PE and mICAM-1 for visualization of integrin activation.

### $G_{\alpha_s}$ -coupled receptor dependence of the effects of sleep (vs. wakefulness) on $\beta_2$ -integrin activation

To demonstrate that the sleep/wake difference in integrin activation was mediated by changes of soluble factors present in plasma acting via  $G_{\alpha_s}$ -coupled receptors, CMV-specific T cells were incubated with plasma collected during sleep or wakefulness in the presence or absence of  $G_{\alpha_s}$ -coupled receptor antagonists or agonists. 80  $\mu$ l of whole blood taken at 6 am from one HLA-A2<sup>+</sup> and CMV-seropositive participant during sleep (to keep endogenous  $G_{\alpha_s}$ -coupled receptor ligands low) was immediately preincubated in the presence or absence of a

composite of the following antagonists: the  $\beta_2$ -AR antagonist ICI-118,551 ( $10^{-7}$  M; Sigma-Aldrich), the PGE<sub>2</sub> EP<sub>4</sub> receptor antagonist ONO-AE3-208 ( $3 \times 10^{-6}$  M; Sigma-Aldrich), and the PGD<sub>2</sub> DP<sub>1</sub> receptor antagonist MK-0524 ( $3 \times 10^{-6}$  M; Santa Cruz Biotechnology) for 5 min at 37°C. These were the antagonists shown to block the effect of the respective agonist on integrin activation (Fig. S1). Blood was then incubated for 5 min at 37°C with 320  $\mu$ l of heparin plasma that had been collected previously from participants during sleep or wakefulness (as described in Participants and study procedure), before the staining with CMV A2-NLV/PE and mICAM-1 as described above. To test whether  $G_{\alpha_s}$ -coupled receptor agonists at low levels corresponding to sleep/wake differences can reverse the effect of sleep on integrin activation, a composite of agonists ((-)-epinephrine [ $10^{-10}$  M], (-)-norepinephrine [ $0.5 \times 10^{-9}$  M], PGD<sub>2</sub> [30 pg/ml], and PGE<sub>2</sub> [30 pg/ml]) was added to the whole blood collected from the CMV seropositive donor and incubated together with plasma derived from sleeping participants for 5 min at 37°C before the staining with CMV A2-NLV/PE and mICAM-1. We used a composite of the  $G_{\alpha_s}$ -coupled receptor antagonists and agonists for these plasma experiments in order to achieve conditions most comparable to those naturally present in the blood during sleep or wakefulness (where several  $G_{\alpha_s}$ -coupled receptor ligands are concomitantly suppressed or increased, respectively). We did not include adenosine or adenosine receptor blockers in these experiments because of the extremely short half-life (<1 s) of (endogenous or exogenous) adenosine in whole blood (Ramakers et al., 2008).

### Statistical analysis

Data are presented as means  $\pm$  SEM. For in vitro data, fitted standard curves were calculated by nonlinear regression using SigmaPlot and analyzed using paired *t* tests. Statistical analyses for the in vivo sleep experiments were generally based on repeated-measures ANOVA with subsequent post hoc contrasts. ANOVA factors were sleep/wake, representing the two experimental conditions; period (night/day); and time, reflecting respective single time points of the night (10 pm to 6 am) and the day (10 am to 6 pm). The differences in baseline measures (at 10 pm) between the sleep and wake conditions were used as covariates to correct for day-to-day variations in immune parameters. Degrees of freedom were corrected using the Greenhouse–Geisser procedure. To identify significant rhythms, Cosinor analysis was performed for the wake and sleep condition with Chronolab using a period of 24 h (Mojón et al., 1992).

### Online supplemental material

Fig. S1 demonstrates that the inhibitory effect of epinephrine, norepinephrine, PGE<sub>2</sub>, and PGD<sub>2</sub> on integrin activation is abolished by  $G_{\alpha_s}$ -coupled receptor antagonists. Fig. S2 shows the effect of epinephrine, norepinephrine, PGE<sub>2</sub>, and PGD<sub>2</sub> on integrin activation in CMV-specific CD8<sup>+</sup> T cell subpopulations.

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